Research Report

A Method for Producing Recombinant Baculovirus Expression Vectors at High Frequency

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ABSTRACT

A system has been developed that can generate recombinant baculovirus expression vectors at frequencies approaching 100%. This system provides a selection for recombinant viruses by using the essential gene downstream of the Autographa californica *nuclear polyhedrosis virus (AcMNPV) polyhedrin expression locus. Two AcMNPV derivatives were constructed in which the expression locus and part of the downstream gene are flanked by restriction sites. The parental viruses are viable; however, restriction of the viral DNAs removes an essential piece of the viral genome. Transfer vectors carry a copy of the missing sequences downstream from the site into which foreign genes are inserted for expression; hence, recombination between a transfer vector and the restricted viral DNA can restore the integrity of the essential gene. Such recombination events also transfer any foreign gene present in the expression locus of the transfer vector to the viral genome. Recombinant viruses therefore have a selective advantage over nonrecombinant viral DNAs. Consequently, a high proportion of the viruses obtained by co-transfecting transfer vector DNA and restricted viral DNA of one of these new viruses expresses the target gene from the transfer vector. This system greatly reduces the time needed to make recombinant baculovirus expression vectors.*

INTRODUCTION

Baculovirus expression systems use viral promoters to drive the expression of foreign genes in insect cells infected with a recombinant baculovirus (3,7–10). These systems frequently produce high yields of proteins due to the strength of the viral promoters. Moreover, insect cells carry out most of the post-translational processing events that occur in mammalian cells so that eukaryotic proteins produced by a baculovirus expression system are usually similar to the authentic protein in their biological activity, structure and antigenicity. This combination of features has contributed to the increasing popularity of baculovirus expression systems.

In the past, one of the disadvantages of these systems was the work involved in isolating a recombinant baculovirus expression vector. The viral genome is too large (130 kb) to manipulate directly, consequently, the standard method for producing virus expression vectors has been to co-transfect insect cells with viral DNA and DNA of a transfer vector modified to incorporate the foreign gene. Recombination *in vivo* can replace a segment of the viral DNA by the modified sequence from the transfer vector. Recombinant viruses are produced but at a low frequency (0.1%–1%). Screening to identify a recombinant virus and separating it from parental virus can therefore involve considerable time and effort. Several modifications of this procedure that facilitate the identification of recombinant viruses by placing a reporter cassette adjacent to the gene to be expressed (19,21,24,25) or that increase the proportion of the viruses that are recombinant (4,12) have been described. Recently, systems have also been developed for generating recombinant baculoviruses in yeast (11) or *in vitro* (13).

Although these advances represent significant improvements over traditional procedures, the utility of the baculovirus expression system would be improved further by a more efficient method for producing recombinant baculovirus vectors. Our objective was to develop a system that would select for the desired recombinants so that most of the viruses released from co-transfected cells would express the target gene.

MATERIALS AND METHODS

Viruses and Cells

Autographa californica nuclear polyhedrosis virus (AcMNPV) C6 (16) and its derivatives, AcRP23-*lacZ* (15), AcRP6-SC (4) and BacPAK5 and BacPAK6, were propagated in *Spodoptera frugiperda* cells (IPLB-SF21) (18) at 28°C using TC-100 insect medium (GIBCO BRL/Life Technologies, Gaithersburg, MD) supplemented with 5% or 10% fetal calf serum (FCS). Procedures for growing AcMNPV derivatives, determining viral titer and analyzing infected cell DNA followed standard methods (3).

Construction of Viruses with *Bsu*36I Sites in ORF603 and ORF1629

Two oligonucleotides, dTGTTAAT TTTCCTGAGGAGA and dACCCTA AGGATTATAAA (nucleotides differing from the wild-type sequence are underlined), were designed to introduce *Bsu*36I sites (CCTNAGG) into ORF603 and ORF1629 of AcMNPV.

Single-stranded DNA of the transfer vector pAcCL29 (6) was mutagenized with these two oligonucleotides using the method of Kunkel et al. (5). Mutagenized pAcCL29 plasmids were screened for the presence of *Bsu*36I sites. Sequencing of one plasmid, pAcCL29#25, confirmed that *Bsu*36I sites had been acquired by the expected sequence changes in ORF603 and ORF1629.

A transfer vector with unique sites for *Mlu*I and *Hin*dIII was made by trimming pAcCL29. First, sequences between the *Acc*I site at 6272 in the *Eco*RI-I AcMNPV DNA [all sequence coordinates are relative to the G of the *Eco*RI site at the left-hand end of the *Eco*RI-I fragment of AcMNPV as base #1 (16)] and the *Nar*I site in the pUC118 (20) segment of the vector were deleted. A second deletion was made between the *Hpa*I site at 2844 in the AcMNPV *Eco*RI-I sequences and the remaining *Pvu*II site in the pUC118 portion of the vector, resulting in pPAK1. The 1.1-kb *Mlu*I-*Hin*dIII fragment of unmutagenized pPAK1 was replaced by the corresponding fragment from pAcCL29#25 carrying the two novel *Bsu*36I sites to make pPAK2. The small *Eco*RV-*Sna*BI fragment of pPAK2 was replaced by the corresponding fragment from pAcEI-I (14) carrying the wild-type polyhedrin promoter and polyhedrin coding sequences. The resulting plasmid, pPAK5, has *Bsu*36I sites in ORF603 and ORF1629 flanking the polyhedrin gene. pPAK6, in which *Bsu*36I sites flank a β-galactosidase gene, was constructed by inserting the 3.74-kb *Bgl*II-*Bam*HI *lac*Z fragment from pCH110-*Bgl*II (15) into the unique *Bam*HI site of pPAK2 in the orientation that would express β-galactosidase from the polyhedrin promoter.

The reporter genes and modified ORF603 and ORF1629 sequences were transferred from pPAK5 and pPAK6 to AcMNPV by co-transfecting the plasmid DNAs with linearized AcRP6-SC viral DNA (4). Recombinant viruses were identified by screening viral plaques for the presence of polyhedra (pPAK5) or a blue color when X-gal was present (pPAK6). Several recombinant plaques were purified, amplified and viral DNAs were extracted. By screening the viral DNAs for the presence of *Bsu*36I sites, a polyhedrin-positive pPAK5 recombinant and a β-galactosidase-positive pPAK6 recombinant that had *Bsu*36I sites in both ORF603 and ORF1629 were identified. These were designated BacPAK5 and BacPAK6, respectively. Additional restriction digests of BacPAK5 and BacPAK6 confirmed that they had the expected structure around the polyhedrin locus (Figure 2, and data not shown).

Restriction of Viral DNA

Viral DNA was extracted from budded virus, purified by banding on cesium chloride gradients and dialyzed against TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) (3). One microgram of viral DNA in 50 µl of 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 µg/ml acetylated bovine serum albumin was incubated with 5 units of *Bsu*36I (New England Biolabs, Beverly, MA) for 2 h at 37°C. After inactivating the enzyme at 70°C for 15 min, the digest was stored at 4°C. Aliquots of the digested and undigested viral DNAs were run on a 0.5% agarose minigel to check that the digest was complete (4).

Co-Transfection of Viral and Transfer Vector DNAs

Thirty-five-millimeter dishes were seeded with 1–1.5 × 10^6 Sf21 cells and incubated at 28°C for 2 to 24 h to allow the cells to attach. One hundred nanograms of *Bsu*36I-digested viral DNA (5 µl), 500 ng of transfer vector DNA in TE and sterile water to make a total volume of 50 µl were mixed in a polystyrene tube. Fifty microliters of 100 µg/ml Lipofectin™ (GIBCO BRL/Life Technologies) were gently mixed with the DNA solution, and the mixture was incubated at room temperature for 15–30 min. Meanwhile, the medium was removed from the cell monolayers, and the cells were washed twice with 2 ml serum-free TC-100 insect medium containing 50 units of penicillin and 50 µg streptomycin per ml. Serum-free TC-100 (1.5 ml) containing antibiotics was added to each dish. The Lipofectin-DNA complexes were added dropwise to the medium covering the cells while the dish was gently swirled. After incubating at 28°C for 5–16 h, 1.5 ml TC-100/10% FCS/antibiotics were added to each dish and the incubation at 28°C continued. Sixty hours after adding the Lipofectin-DNA complexes to the cells, the medium containing viruses released by the transfected cells was transferred to a sterile container and stored at 4°C.

Recombination Assays

A standard plaque assay procedure (3) was used to obtain viral plaques from dilutions of the media harvested from the co-transfections. Plaques were stained by adding 100 µl 0.08% neutral red, 0.2% X-gal in phosphate-buffered saline (PBS) (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) to the medium in each dish and incubating at 28°C for 5–8 h. The stain was removed, the dishes inverted and left at room temperature for 16 h. Individual plaques were examined under a microscope and scored for the presence or absence of polyhedra and blue or white color.

RESULTS

Design of Viruses That Allow Selection for Recombinants

The finding that linearized AcMNPV DNA retained the ability to recombine with homologous viral sequences in transfer vectors (4), suggested a scheme that would permit a selection for recombinant viruses. The principle is to place a gene, or part of a gene, which is essential for viral replication between two restriction sites that do not occur elsewhere in the viral DNA. Restriction of the viral DNA would release a small fragment carrying essential sequences (Figure 1). If the large fragment, carrying most of the viral genome, recombines with a transfer vector carrying an intact copy of the essential gene, the resulting circular viral DNA would contain all the genes necessary for viral replication and would give rise to viable virus particles (Figure 1). The same recombination events that restore the integrity of the essential gene would transfer the target gene from the transfer vector to the viral genome (Figure 1). If the large viral

fragment were to recircularize by itself, the resulting viral DNA would lack an essential part of the genome and would not give rise to viable viruses. Recombinant virus expression vectors would thus have a selective advantage over nonrecombinant viral DNAs.

In the AcMNPV genome, the open reading frame downstream from polyhedrin, ORF1629, appears to be essential. Insertions into the *Sna*BI site in the C-terminus of this gene produce viruses that can only replicate in the presence of a helper virus (16). All AcMNPV transfer vectors based on the polyhedrin locus contain the C-terminus of ORF1629; therefore, this gene provides a convenient essential gene for use in the scheme outlined above. Scanning the sequence of ORF1629 (16) revealed several locations at which a site for *Bsu*36I, an endonuclease that does not cut wild-type AcMNPV DNA (4), could be introduced by changing one or two nucleotides. The essential nature of ORF1629 dictated that the necessary changes should not alter the amino acid sequence of the ORF1629 protein. It was also desirable that the length of the region in which recombination could occur should be maximized and that the ends created by *Bsu*36I cleavage should match the wild-type ORF1629 sequences perfectly so that recombination with the transfer vector would not be blocked. Changing the T at position #5564 in the *Eco*RI-I sequence (16) to a C would create a *Bsu*36I site satisfying these criteria. Similar analysis of the sequences 5′ to the polyhedrin promoter identified a *Bsu*36I site that could be created within ORF603 by changing the TA at positions #4163 and #4164 to GG. ORF603 is not essential for virus growth in cell culture (1), hence the replacement of a tyrosine residue in the putative ORF603 protein by a proline as a result of these changes should be of no consequence. Cleavage at the *Bsu*36I site would also create an end that is perfectly homologous to the wild-type ORF603 sequence.

Construction of Viruses Having an Essential Segment Flanked by *Bsu*36I Sites

AcMNPV derivatives designed to use ORF1629 as a positive selection were constructed by placing one site for *Bsu*36I within the C-terminus of ORF1629 and another site within ORF603 on the opposite side of the polyhedrin locus. Oligonucleotide mutagenesis (5) of the transfer vector pAcCL29 (6) was used to create *Bsu*36I sites in ORF603 and ORF1629 at the locations specified. The region spanning the mutations was sequenced and then recloned into an unmutagenized transfer vector (pPAK1) to generate pPAK2. To allow plaques of the new viruses to be recognized by a simple visual screen, the gene for polyhedrin or β-galactosidase was inserted downstream of the polyhedrin promoter in pPAK2 to make pPAK5 and pPAK6, respectively.

Viruses that had incorporated the marker gene and the two flanking *Bsu*36I sites from pPAK5 or pPAK6 were isolated from the products of recombination between the transfer vector and linearized AcRP6-SC viral DNA. The structure of these AcMNPV derivatives, designated BacPAK5 and BacPAK6, is shown in Figure 2A. Restriction of the viral DNA from BacPAK5 with *Bsu*36I yields a large fragment carrying most of the viral genome (127 kb) and a 1.4-kb fragment (Figure 2B). Digestion of BacPAK6 viral DNA yields the same large fragment and two small fragments, 1.1 kb and 3.3 kb, due to the additional *Bsu*36I site within the *lac*Z gene (Figure 2B). Digestion of BacPAK5 and BacPAK6 viral DNAs with *Bgl*II in addition to *Bsu*36I mapped the *Bsu*36I sites to the expected locations in the viral genome (data not shown).

BacPAK5 and BacPAK6 gave normal yields of virus particles when propagated in tissue culture (data not shown) indicating that the base changes in ORF603 and ORF1629 had no deleterious effect on virus replication or infectivity. The polyhedra positive or β-galactosidase positive phenotype of these viruses was stable for at least 6 passages in cell culture.

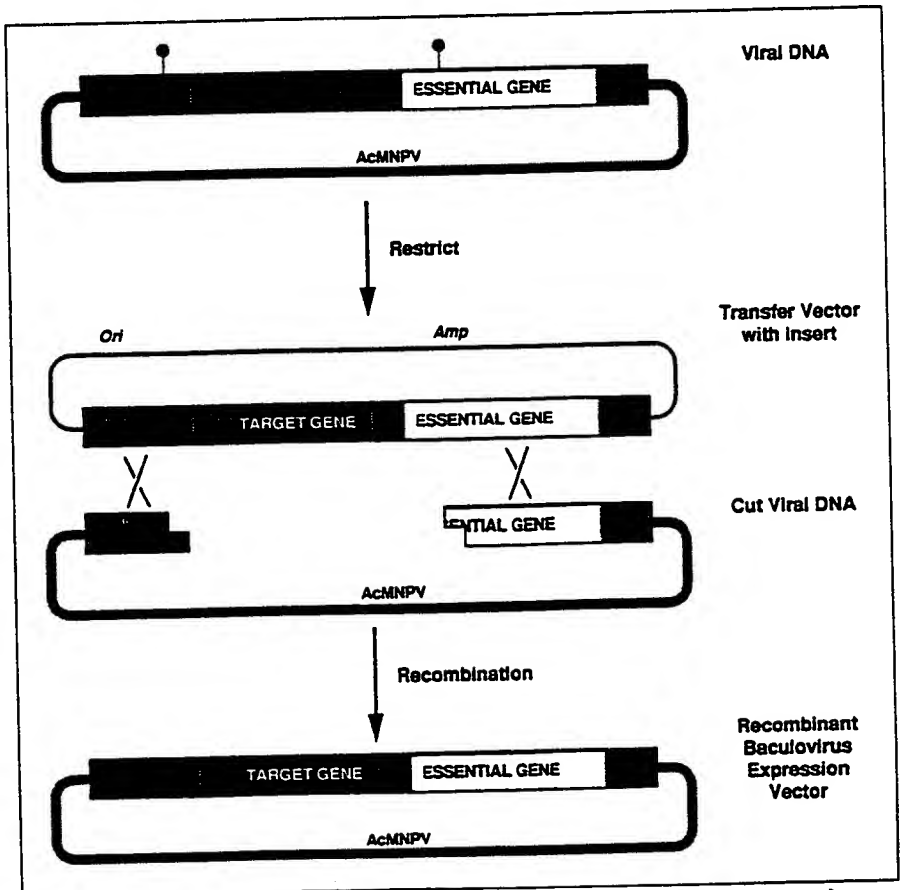

Figure 1. A scheme for positive selection of recombinant baculoviruses. ↑restriction site, ▶ polyhedrin promoter.

Table 1. Recombination Between Restricted Viral DNAs and Transfer Vectors

Viral DNA Digest	Transfer Vector	Number of Transfections	Plaque Phenotype of Progeny Viruses				Recombinant as % of Non-Parental	
			Parental	Recombinant	Mixed	Neither	Median	Range
AcRP23-*lacZ*	pAcEI-I	6	23%	28%	1%	55%	36%	(26–44)
BacPAK5	pAcRP23-*lacZ*	9	37%	43%	6%	8%	81%	(67–96)
BacPAK6	pAcEI-I	9	0%	95%	0%	5%	95%	(86–99)

Circular DNA extracted from virus particles was restricted with *Bsu*36I; AcRP23-*lacZ* DNA has a unique *Bsu*36I site, BacPAK5 has two *Bsu*36I sites and BacPAK6 has three *Bsu*36I sites (Figure 2A). 100 ng of viral DNA were transfected into *S. frugiperda* cells with 500 ng of transfer vector DNA using Lipofectin. Plaques of the viruses released from the transfected cells were scored for viral and transfer vector markers. For the viruses AcRP23-*lacZ* and BacPAK6, the marker is β-galactosidase, detected as blue plaques in the presence of X-gal; for BacPAK5 the viral marker is polyhedrin, detected by the presence of polyhedra in the plaque. For the transfer vector pAcRP23-*lacZ*, the marker is β-galactosidase, and for pAcEI-I, the marker is polyhedrin. Results are presented as the median of 6 or 9 independent transfections.

"Recombinant as % of non-parental" is calculated as number of recombinant plaques/(number of recombinant plaques + number of mixed plaques + number of plaques expressing neither marker) × 100%, for each transfection experiment.

Recombination Between Restricted BacPAK5 and BacPAK6 Viral DNAs and Transfer Vectors

The utility of BacPAK5 and BacPAK6 for the generation of recombinant viral expression vectors was compared with a virus, AcRP23-*lacZ*, which contains a single *Bsu*36I site and is used in the linear viral DNA method of making recombinant viruses (4). Recombination was assayed by transfecting *S. frugiperda* cells with DNA of a plasmid transfer vector and viral DNA digested with *Bsu*36I. The transfer vector carried a marker that could be detected by visual screening of viral plaques and distinguished from the marker carried by the viral DNA. If the viral DNA carried the polyhedrin gene (BacPAK5), a transfer vector carrying the *lacZ* gene was used. Conversely, if the viral DNA carried *lacZ* (AcRP23-*lacZ* or BacPAK6), a transfer vector

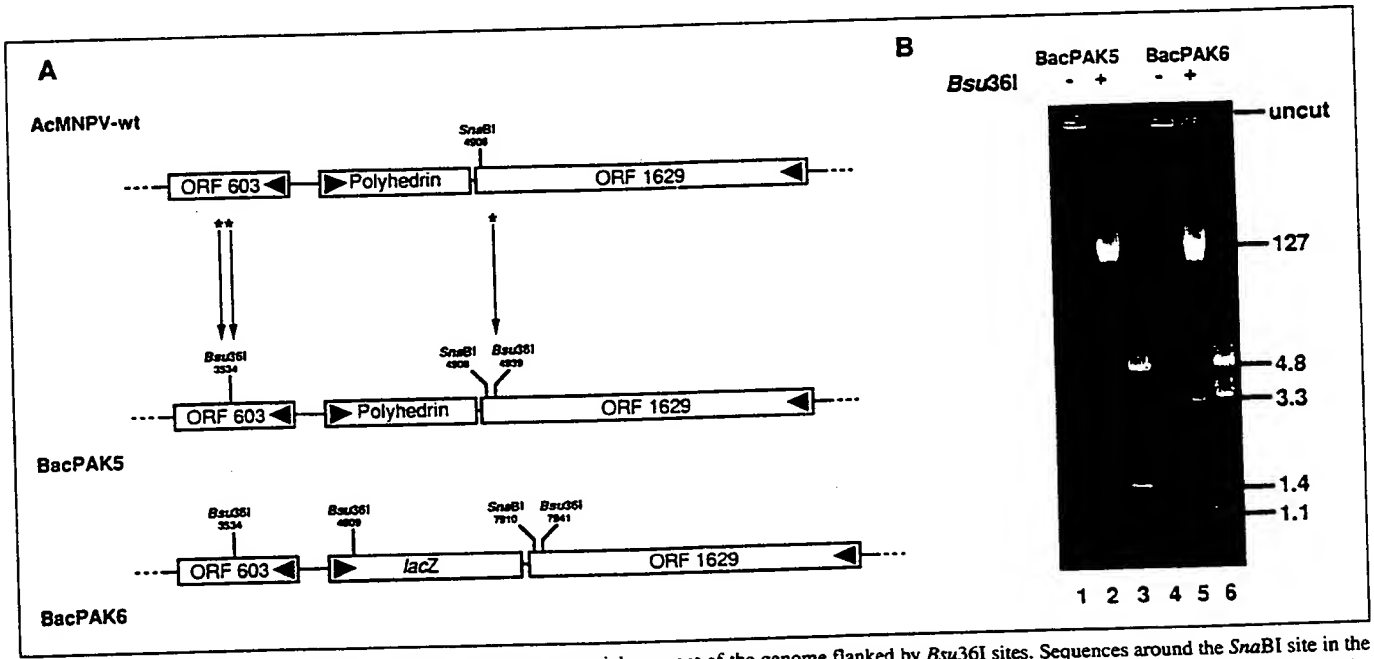

Figure 2. A. Construction of AcMNPV derivatives having an essential segment of the genome flanked by *Bsu*36I sites. Sequences around the *Sna*BI site in the C-terminus of ORF1629 are essential for virus replication (16). Site-directed mutagenesis was used to create sites for *Bsu*36I flanking this region and either the polyhedrin gene of AcMNPV or *lacZ*. The *Bsu*36I sites in the resulting viruses differ in the central degenerate nucleotide: CCTCAGG in ORF603, CCTGAGG in *lacZ* and CCTTAGG in ORF1629. Coordinates are relative to the G of the *Eco*RI site at the left-hand end of the *Eco*RI-I fragment of AcMNPV as base #1 (16). (The *lacZ* gene is not to scale.) B. Restriction of BacPAK5 and BacPAK6 viral DNAs with *Bsu*36I. BacPAK5 and BacPAK6 viral DNAs were digested with *Bsu*36I and run on a 0.5% agarose gel (lanes 2 and 5) alongside untreated viral DNAs (lanes 1 and 4). Markers were provided by *Bsu*36I digests of the progenitor plasmids pPAK5 (lane 3) and pPAK6 (lane 6) and contained a 4.8-kb plasmid vector fragment in addition to the small *Bsu*36I fragments. Uncut (circular) viral DNA did not enter the gel. Digestion with *Bsu*36I produced a 127-kb fragment from both viruses and either a 1.4-kb fragment (BacPAK5) or 3.3-kb and 1.1 kb fragments (BacPAK6).

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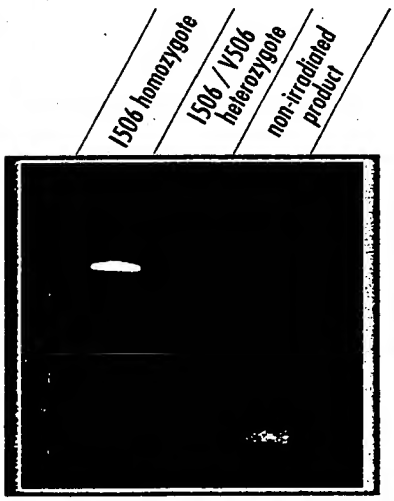
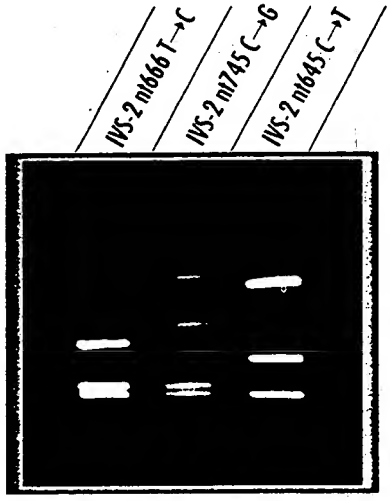
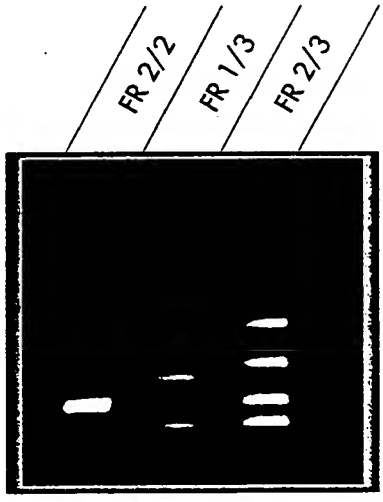


DGGE analysis of the I506V polymorphism in exon 10 of the CFTR gene, using ChemiClamped primers



DGGE analysis of exon 3 (and flanking regions) of the human ß-globin gene showing different intron 2 (IVS2) nucleotide changes (heterozygous subjects).



DGGE analysis of ß-globin frameworks (FR) 1, 2 and 3 using ChemiClamped primers.

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carrying the polyhedrin gene was used. Viruses released from the co-transfected cells were plated out and scored for the presence of the viral and transfer vector markers. Four different plaque phenotypes can be distinguished: (i) parental plaques expressing the marker from the viral DNA but not the transfer vector marker, (ii) recombinant plaques expressing the marker from the transfer vector instead of the viral marker, (iii) plaques expressing both markers—these could be due to a virus that incorporated the transfer vector marker without losing the viral marker gene or due to coinfection with a parental and recombinant virus, and (iv) plaques that express neither the viral nor the transfer vector markers.

In most procedures for isolating a recombinant AcMNPV expression vector, plaques that express the marker gene from the parental viral DNA can be identified by a simple visual screen and rejected. The best measure of the utility of the new viruses is therefore the fraction of non-parental virus plaques that are recombinant. Using this measure, the benefit of the two new viral DNAs is readily apparent (Table 1); using BacPAK5 DNA, a median of 81% of the non-parental plaques are recombinant, for BacPAK6 DNA the ratio is even higher at 95%, compared with 36% for the singly cut AcRP23-*lac*Z viral DNA.

The majority of viruses produced from linearized AcRP23-*lac*Z DNA express neither the gene from the transfer vector nor the marker gene from the parental virus (Table 1). These plaques probably contain viruses with deletions in *lac*Z arising from unfaithful recircularization of the linearized viral DNA (4). This class of viruses is greatly reduced when restricted BacPAK5 or BacPAK6 viral DNAs are used in the co-transfection providing evidence that the ORF1629 selection against non-recombinant viruses is effective.

The total yield of virus recovered from co-transfections using restricted BacPAK5 or BacPAK6 DNA was very low; if our results were based on a few recombination events, the recombination frequencies obtained could have been biased. To validate our results, we overlaid the cells with agarose 6 h after transfection so that each transfected cell that produced virus would give rise to a focus of infection. Five days later, 100–200 foci of infection were visible in each dish (data not shown). Each recombination frequency we determine is therefore based on a pool of viruses produced from more than 100 independently transfected cells.

DISCUSSION

We had previously described how linearization of baculovirus DNA before co-transfection with transfer vectors could be used to boost the proportion of progeny viruses that were recombinant to about 30% (4). In these experiments, the background of nonrecombinant viruses came from two sources: (i) viral DNA molecules that did not get cut by the restriction enzyme [a small fraction of uncut DNA can lead to a significant background of nonrecombinant viruses because circular viral DNA is 15-fold–150-fold more infectious than linear DNA (4)], and (ii) linearized viral DNA molecules that recircularized inside the insect cell without recombining with a transfer vector. To eliminate the type (ii) background, two viruses were constructed so that restriction of the viral DNA would separate part of the essential gene downstream of the polyhedrin expression locus from the remainder of the viral genome. Even if the large fragment of viral DNA recircularized, the resulting virus genome would lack essential sequences and would not be able to generate viable viruses, whereas a double recombination between the large fragment of viral DNA and homologous sequences on a transfer vector would generate a circular viral genome with an intact copy of the essential gene that could produce viable viruses. We expected that the presence of two or three restriction sites in the viral DNA would also reduce the type (i) background because the fraction of molecules that had not been cut at least once would be extremely small. One of our novel AcMNPV derivatives, BacPAK6, fulfills these expectations; 85%–99% of the viruses obtained by co-transfecting restricted BacPAK6 viral DNA with DNA of a transfer vector expressed the gene from the transfer vector.

The use of restricted BacPAK6 viral DNA to generate recombinant AcMNPV expression vectors yields a higher proportion of recombinant viruses than existing methods. This system has the additional advantage of being compatible with a wide range of existing vectors. The ORF1629 sequences required to rescue restricted BacPAK6 viral DNA are present in most transfer vectors that are based on the polyhedrin locus, including vectors containing a secretion signal (17), vectors for producing two proteins simultaneously (22–24) and vectors containing alternative promoters (2). In contrast, other methods require the use of specially constructed transfer vectors, which are often cumbersome due to the presence of a reporter gene.

The availability of a system that can generate recombinant baculovirus expression vectors at frequencies of close to 100% should reduce the time taken to isolate recombinant viruses, facilitate studies that involve the expression of large numbers of variant proteins and encourage more researchers to try this excellent expression system.

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